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Artificial selection on male genitalia length alters female brain size

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Abstract

Male harassment is a classic example of how sexual conflict over mating leads to sex-specific behavioural adaptations. Females often suffer significant costs from males attempting forced copulations, and the sexes can be in an arms race over male coercion. Yet, despite recent recognition that divergent sex-specific interests in reproduction can affect brain evolution, sexual conflict has not been addressed in this context. Here, we investigate whether artificial selection on a correlate of male success at coercion, genital length, affects brain anatomy in males and females. We analyzed the brains of eastern mosquito fish *Gambusia holbrooki*, which had been artificially selected for long or short gonopodium, thereby mimicking selection arising from differing levels of male harassment. In analogy to how prey species often have relatively larger brains than their predators, we found that female, but not male, brain size was greater following selection for a longer gonopodium. Brain subregion volumes remained unchanged. These results suggest that there is a positive genetic correlation between male gonopodium length and female brain size, which is possibly linked to increased female cognitive ability to avoid male coercion. We propose that sexual conflict is an important factor in the evolution of brain anatomy and cognitive ability.

Key words: Sexual conflict, sexual dimorphism, brain evolution, male harassment, gonopodium, *Gambusia holbrooki*.

Introduction

Evolutionary conflicts of interest between the sexes are ubiquitous, inevitable and rooted in anisogamy [1,2]. Conflicts may create divergent selective pressures for males and females, driving the conspicuous sexual dimorphism in morphology and behavior observed in many species [3,4]. Different interests in reproduction might, however, also account for sex differences in the nervous system [5-10]. Males, for example, may have larger hippocampal size and be better at spatial navigation - important when locating a potential mate [11-16]. Females, on the other hand, often have to remember and compare numerous earlier encounters with males to make an adaptive mate choice decision [17-19]. Mating decisions can therefore place distinct demands on cognitive abilities in both sexes. Here we adhere to the broad definition of “cognition” as the acquisition, processing, retention and use of information [20]. Because cognition is intimately linked to brain anatomy [21-24], cognitive demands of reproduction should lead to enhancement of particular brain structures, and select for increased brain size.

Male sexual harassment, where males force females to mate with them, is a classic example of how conflict over mating can shift the balance of sexual conflict in favor of males [25]. In a non-coercive mating system, males are under strong selection to evolve secondary sexual traits to exploit pre-existing female preferences, inducing females to mate in a suboptimal manner. Under male coercion, however, selection to avoid the reproductive interest of males should act strongly on females to avoid the potential costs associated with a sub-optimally high mating rate. Indeed, females can suffer significant costs from multiple mating (e.g. physical damage, disease transmission) and male harassment (e.g. reduced feeding efficiency, increased risk of predation and lower offspring survival) [26-29]. To keep harassment at bay, females may thus develop cognitive tools that enable them to predict or detect the presence of males sooner, and

thereby escape mating attempts. The interactions between force-copulating males and females trying to avoid male coercion may, in many aspects, resemble the dynamics of predator prey interactions, which are thought to be cognitively demanding for both actors - large-brained predators tend to prey on large-brained prey ([30], and see also [31-33]). One evolutionary outcome of sexual coercion could be a cognitive arms race between the sexes to facilitate and subvert male coercion attempts respectively [34,35]

Recent hypotheses describing brain evolution in response to conflict over reproduction are formulated primarily for mating systems characterized by female mate choice [5-7,9,10,36]. Here we test how sexual conflict and male coercion affect the brain anatomy of both sexes. We used eastern mosquitofish (*Gambusia holbrooki*), which had been artificially selected for the length of the external male genitalia (the gonopodium). This selection for greater or shorter gonopodium length should mimic selection arising from environments with stronger or weaker male harassment in terms of male coercion rates. In this species males do not generally court females [37,38], and most inseminations occur through forced copulations. In *G. holbrooki*, males with a naturally longer gonopodium have greater reproductive success (Head et al. preprint; Vega Trejo et al. preprint; but see [39]). There is also evidence in another Poeciliid fish, the guppy, that males with a longer gonopodium have higher mating rates because a long gonopodium facilitates genital contact with females [40]. We assume that there is natural selection against greater gonopodium length because it potentially reduces male swimming performance (e.g. burst swimming speed [41]; but see [39]).

If, as in predator-prey interactions, males and females are in a cognitive arms race over male coercion, we might predict larger brains in both sexes when there is selection for longer gonopodia. Females in our selection lines were assigned a mate; we therefore

expect any potential effect of male gonopodium length selection on female brain anatomy to reflect a genetic correlation present in the mosquitofish genome due to past selective pressures acting in the wild. This assumption is supported by recent data as populations with long gonopodia also have higher levels of reproductive success (Head et al. preprint; Vega Trejo et al. preprint), an effect that is likely to be driven by higher levels of successful coercive matings in such populations. But there may also be costs associated with evolving longer gonopodia, and these could potentially influence investment into the brain, which is a highly costly organ to develop and maintain [24,42-45]. For instance, longer gonopodia have been shown to hamper swimming ability ([41], but see [39]); or there might be a direct life history trade-off between costly sexual traits (gonopodium length) and expensive brain tissue [46]. Hence, it is possible that males in lines selected for a longer gonopodium might decrease investment into neural development, resulting in the evolution of smaller brains.

The hypotheses above give clear, albeit in the case of males opposing, predictions about how selection on gonopodium length (and, by extension, male coercion) will affect male and female brain size. It is more difficult to make accurate predictions for brain subregion volumes as the functions of different regions are still only partly understood and sometimes have multiple functions [47]. However, the cognitive centre, the telencephalon, potentially together with the visual centre in the brain, the optic tectum, could be of high importance in manipulating the reproductive interests of the other sex. If so, the telencephalon and/or optic tectum should be larger in the lines selected for a longer gonopodium. For the other regions we make no predictions and treat our analysis as a way to identify regions of the brain that are potentially affected by selection on male coercion.

Methods

Gonopodium selected mosquitofish

The fish used here were offspring of mosquitofish (*Gambusia holbrooki*), which had previously been selected for long and short gonopodium length [39]. Briefly, fish collected in Sydney, Australia in 2007 were used to set up three replicates (A, B, C), each consisting of two selection lines ('up' - and 'down' - selected on gonopodium length) and an unselected 'control' line. Sires to initiate the selection lines were chosen by measuring standard body length (SL) and gonopodium length in wild caught males. The top 30-40 males with the largest ('up') and shortest ('down') relative gonopodium length respectively were then paired with two virgin females that were lab-reared offspring of gravid wild-caught females. Another randomly chosen 30-40 wild-caught males were used to start up the 'control' lines. Pairing each male with two virgin females increased the likelihood that all males sired offspring. Ten juveniles per male were then pooled and reared communally with other fry from the same experimental line until the offspring were separated by sex at the first sign of maturity. Males were isolated until fully matured, immobilized in ice water and photographed to measure their standard length and gonopodium length. From generation two to eight, males from the selection lines (n= 40 of about 129 measured males per experimental line) were chosen based on their deviation (positive residuals for 'up' and negative residuals for 'down') from a regression line of log gonopodium length on log standard length. These males were then paired to two unrelated virgin females from the same line. As described above, the offspring were then reared communally until juveniles could be separated by sex and relative gonopodium length was measured in adult males (for details of sample sizes see [39]). After reproducing, all 40 males and females that produced generation nine were euthanized with an overdose of anaesthetic (clove oil in alcohol) and preserved in paraformaldehyde. Before the brains were dissected out all fish were

washed in phosphate buffer (PBS) and their standard length was measured to the nearest 0.01 mm using a digital caliper.

Dissections and measuring of brain regions

The brains of 180 fish from the selection lines (n = 90 males, n = 90 females; N=10/replicate/experimental line) were dissected out under a stereomicroscope and weighed to the nearest 0.001 mg. Volumes of brain subregions (olfactory bulb, telencephalon, optic tectum, cerebellum, dorsal medulla, hypothalamus) were quantified according to the method described in detail in [8]. Briefly, we used a dissection microscope (LAS EZ version 3.0, Leica Microsystems Switzerland) to obtain four digital images (ventral, dorsal, right and left side) per brain. We then measured the length L , width W and height H of the respective brain subregions (Image J version 1.48v) and calculated the volume of all brain subregions by applying the mathematical formula for an ellipsoid model: $V = (L \times W \times H) \pi/6$ [48]. For paired regions (optic tectum, telencephalon, olfactory bulb) we measured both sides and added the volumes to obtain a total region volume. All dissections and digital image analyses were done by the same person (SDB) blind to the treatment.

Statistical analysis

To analyse how selection on gonopodium length affected brain size, we used a linear mixed-effect model (LMM) on data of all fish (overall; males and females). This model included brain weight as the dependent variable, sex (male, female) and experimental selection ('up', control, 'down') as fixed factors, body size (standard length) as a co-variate; and replicate (A, B, C) nested in experimental selection as a random factor. To test how selection on gonopodium length affected the male and female brain

respectively, we performed two additional analogous LMMs. Brain weight and standard length were log10 transformed prior to analyses.

Due to sex differences in both brain size and brain-body allometry (see results) we investigated selection-specific changes in brain subregion volumes (olfactory bulbs, telencephalon, optic tectum, cerebellum, hypothalamus and dorsal medulla) in males and females separately. We used two analogous multivariate mixed effect models that included experimental selection as fixed factors; total brain weight as a co-variate, and replicate nested within experimental selection as a random factor. As for the above brain size analyses, both brain volume and brain components were log10 transformed prior to analyses.

All models were performed using R statistical software v 3.2.0 [49]. LMMs were fitted using the *lmerTest* package v. 2.0-25 with *p*-values calculated based on Satterthwaite's approximation for denominator degrees of freedom. Pairwise comparisons between selection lines were conducted posthoc, using the *diffsmeans* function within the *lmerTest* package [50]. Multivariate mixed effect models were fitted using a Bayesian approach implemented in the R package *MCMCglmm* v 2.21 [51], a reasonable approach to assess statistical significance of fixed effects in models that use nested random terms. Flat priors were used for the fixed effects and locally uninformative priors were used for random effects, both representing little prior knowledge. We defined 1000 burn-in iterations, followed by 500000 iterations sampled with a thinning interval of 500, resulting in a sample size from the posterior distribution of 1000. All autocorrelations across successively stored posterior samples were in the interval <0.1 and >-0.1 . The default contrast matrix in R (“Contrast treatments”) was used to generate the parameter estimates. Significance of the fixed effects was inferred if the 95% CI of

the posterior distribution did not cross zero, and the P -values were equal to or less than 0.05. Model selection was done by backwards elimination of non-significant terms [52] based on Akaike's information Criterion ($\Delta \text{AIC} < 2$) and was confirmed by automated model selection applying the *glmulti* R package [53]. For multivariate analyses the best model was selected based on the Deviance Information Criterion (DIC). As for AIC, a smaller value of DIC indicates a better model fit. If interaction terms are not presented they did not have a significant effect on brain weight, or on the volume of brain subregions. All summary statistics are presented as mean \pm SE.

Results

There was no general effect of selection on gonopodium length on brain size (LMM; Selection: $F_{2,9} = 1.482$, $p > 0.1$, table 1), but there was a non-significant trend towards sex-specific effects on brain size (selection * sex; $p = 0.062$). As expected, large fish had bigger brains (LMM; Body size: $F_{1,180} = 73.543$, $p < 0.001$, table 1). However, females had a smaller relative brain size than males, which may be explained by sex differences in allometry (figure S1), potentially caused by the narrower range of variation in body size among females than males that we used in this study (LMM; Sex: $F_{1,178} = 9.674$, $p = 0.002$, Sex * Body size: $F_{1,178} = 9.832$, $p = 0.002$, table 1).

We therefore investigated the sex-specific trends in more detail using within-sex analyses. Females in the lines selected for a longer gonopodium had larger relative brain size than females in the lines selected for a shorter gonopodium and control females (LMM; Selection: $F_{2,90} = 3.611$, $p = 0.031$, pairwise comparisons: up vs. down: $t = -2.37$, $df = 90$, $p = 0.02$, up vs. control: $t = -2.25$, $df = 90$, $p = 0.03$; table 1, figure 1b). Up-selected line females had, on average, a 6.5% heavier brain (up-selected females: 6.59 ± 0.08 mg) than down-selected line females (down-selected females: 6.19 ± 0.10 mg), and

a 4.6% heavier brain than females from the control lines (control females: 6.30 ± 0.10 mg). Selection on gonopodium length did not, however, affect relative male brain size (LMM; Selection: $F_{2,90} = 0.710$, $p > 0.1$; table 1, figure 1a).

Finally, we did not detect any effect of selection on gonopodium length on the volume of any particular brain subregion for either sex (MCMCglmm; $p > 0.1$ for all six subregions tested, table S1, figures S2 a-f).

Discussion

We used artificial selection on gonopodium length in mosquitofish to mimic the selection pressures that act in environments favouring different levels of male coercion success (i.e. more coercion selects for a longer gonopodium). We found that female, but not male, brain size increased in lines selected for a longer gonopodium.

At first, this sex difference seems surprising. Similarly to the cognitive co-evolution reported between predator and prey species, we expected the sexes to be in a cognitive arms race over male coercion attempts. Since larger brains confer better cognitive abilities [21-24] this should have resulted in the evolution of larger male and female brains, but this was not corroborated by our results. Instead, only females from the lines with males with a longer gonopodium had significantly larger brains (6.5%) than females from lines with males with a shorter gonopodium. This is congruent with prey - here the females escaping male coercion - sometimes having strong selection on brain size [30]. We interpret our result as support for the hypothesis that, at least for females, cognition plays a crucial role in the conflict over mating decisions [5,6].

The acquisition of better cognitive abilities, via increased brain size, may enable females to exert greater control over the paternity of their offspring, even when coercive mating is the only tactic adopted by males. In *G. holbrooki*, males usually sneak up to females, attempting up to a thousand forced copulations per day [37]. In such a scenario gonopodium size should predict mating success. This was found in two large-scale studies on *G. holbrooki* males with natural variation in gonopodium length. Males with a longer gonopodium had higher reproductive success (Head et al. preprint; Vega Trejo et al. preprint). Although such an effect was not apparent when gonopodium-selected males were exposed to wild-type females [39], our finding nevertheless suggests that selection for gonopodium size increases male coercive mating performance in the wild. A genetic correlation between selection on male gonopodium size and female brain size would be difficult to explain if it were not mediated by an interaction between the sexes. A larger brain might allow females to better predict their environment, detect males earlier and/or have faster reactions to more successfully evade males. Females with better cognitive skills may even exert mate choice by escaping the mating attempts of certain males.

We found no evidence that any particular brain region in either sex responded to artificial selection on gonopodium length. In this context our results support the ‘concerted evolution hypothesis’, that all brain regions in concert drive the development of larger brains [54,55]. The cognitive processes that enable females to express mate choice in a mating system characterized by male coercion seemingly demand the integration of a broad range of neuronal processes and, as suggested previously, are not limited to separate areas of the brain that process sensory information [9].

Artificial selection on gonopodium length in male mosquitofish did not result in males with longer gonopodia having larger brains or *vice versa*. This finding primarily suggests that male cognitive skills do not play a prominent role in determining the outcome of forced mating attempts. Gonopodium size is unlikely to be traded off against expensive brain tissue because we find no negative association between gonopodium size and any aspect of brain anatomy in males. Of course, we cannot rule out that the constant on-going costs of decreased swimming performance associated with growing a longer gonopodium ([41]; but see [39]) restrain selection for a larger brain in long-gonopodia males. As a result we might not detect any difference in brain size between long- and short-gonopodia males. However, it is difficult to see how the cognitive skills used to evade or pursue coercive mating would greatly differ between the sexes. Females should suffer significantly more from being forced into copulation than males do by losing an opportunity to mate [56,57]. We therefore speculate that females are under stronger selection to invest in costly traits, like larger brains, to evade male coercion.

In a recent study on guppies (*Poecilia reticulata*), which are close relatives to mosquitofish, Kotrschal et al [58] found a positive genetic correlation between gonopodium length and male brain size. Given that selection for a longer gonopodium in *G. holbrooki* did not lead to a correlated response in male brain size, it seems unlikely that brain size and male sexual traits are generally positively linked in Poeciliid fish. We speculate that if a positive genetic correlation between gonopodium length and brain size does exist, it is more likely to evolve in species where males engage in both mating tactics, *i.e.* courting females and forcing females to mate with them (as seen in guppies, [58]). The logic behind this reasoning is based on the tight link between cognitive abilities and brain size [21-24] and that more intelligent males (*i.e.* ones with

larger brains) are generally more attractive to females [59-61]; they therefore gain more mating opportunities, while a longer gonopodium should aid in overcoming mate assessment, thereby facilitating male coercion [40].

In the absence of male coercion, when females choose whom to mate with, both the male and female brain should be selected for increased size, because not only choosing but also courting a mate can be cognitively demanding ([36,60]; Corral-Lopez et al. unpublished data). In *G. holbrooki* male coercion is, however, the only strategy adopted by males. Only female brain size increased following strong directional artificial selection on gonopodium length. Therefore we hypothesize that greater sexual dimorphism in relative brain size is a function of male harassment and forced copulations. Poeciliid fishes and, in particular, the guppy (*Poecilia reticulata*) could offer a valuable opportunity to study this question as this species shows a pronounced sex-ratio continuum in the wild [62]: from female-biased populations where female mate choice should be prevalent, to male-biased populations where male coercion is more frequent [63].

To conclude, selection on gonopodium length did not reveal evidence for a cognitive arms race between the sexes. Instead, our results support a scenario where female cognition evolves in response to the sexual conflict favouring male genital size evolution. We propose that sexual conflict driven by male coercion is an important factor in brain anatomy evolution, and with this study we provide a prospect for future work on brain evolution in relation to different mating strategies.

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Author contributions

MDJ and IB generated the gonopodium selection lines, SDB dissected the brains and performed the analyses. All authors contributed to writing the paper.

Ethical statement

All procedures were approved under Australian National University Animal Ethics (Permits F.BTZ.90.05, F.BTZ.26.08 and A2011/64) and New South Wales DPI Scientific Collection regulations (Permit P06/0147-1.0).

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Data accessibility

All data will be deposited on Dryad upon acceptance.

Competing interest

We have no competing interests to declare.

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Table 1: Results from the best models (LMM's) on brain weight including selection on gonopodium length as fixed factor, body size as co-variate and replicate nested within selection as random effect. The response variable brain weight and the co-variate body size were log-transformed prior to analyses. Statistically significant results ($p < 0.05$) are highlighted in bold. If interaction terms are not presented they did not have a significant effect on brain size.

	Effects	<i>df (approx.)</i>	<i>F</i>	<i>p</i>
Overall	Selection	2/9	1.482	0.276
	Sex	1/178	9.674	0.002
	Body size ¹	1/180	73.543	<0.001
	Sex*Body size	1/178	9.832	0.002
Males	Selection	2/9	0.710	0.551
	Body size ¹	1/173	896.0	<0.001
Females	Selection	2/90	3.611	0.031
	Body size ¹	1/90	6.103	0.015

¹ log transformed Standard length

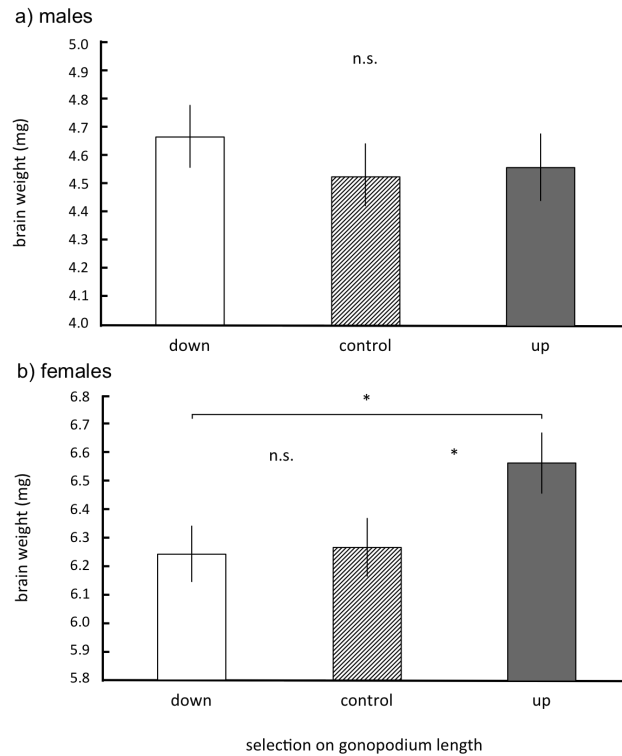


Figure 1: The brain weight of (a) male and (b) female mosquitofish ($n = 90$ for each sex) that were selected for relative gonopodium length (down, control, up) over eight generations. Shown are the model estimates (mean \pm SE) from a model that used brain weight (non-log transformed values) as response variable, experimental selection as a fixed factor, body size as a co-variate and the three independent replicates nested in experimental selection as a random factor (see methods). Note that scaling for the Y-axis differs for male and female brains.

Table S1: Results from Bayesian statistical analyses of the effect of selection on gonopodium length on male and female brain subregion volumes. Posterior means show the differences between the group means of experimental selection lines. For the relationship between the total brain weight and the respective brain subregion, the posterior mean indicates the slope of the regression. Statistically significant results ($p < 0.05$) are highlighted in bold. Note that *p-values* are not corrected for multiple testing.

Model	Response variable (Brain subregion)	Parameter	Post. mean	95% credible interval	<i>P</i> MCMC
Males	telencephalon	Brain weight	1.099	(0.05, 2.11)	0.022
		Selection Up vs. Down	0.009	(-0.26, 0.25)	0.904
		Selection Control vs. Down	0.016	(0.24, 0.25)	0.866
		Selection Control vs. Up	0.015	(-0.25, 0.27)	0.916
	optic tectum	Brain weight	0.810	(-0.39, 1.84)	0.156
		Selection Up vs. Down	0.009	(-0.26, 0.24)	0.916
		Selection Control vs. Down	0.006	(-0.26, 0.26)	0.964
		Selection Control vs. Up	-0.003	(-0.25, 0.26)	0.984
	cerebellum	Brain weight	1.148	(0.13, 2.25)	0.032
		Selection Up vs. Down	0.0152	(-0.23, 0.26)	0.922
		Selection Control vs. Down	0.023	(-0.23, 0.24)	0.840
		Selection Control vs. Up	0.007	(-0.25, 0.24)	0.958
	dorsal medulla	Brain weight	0.870	(-1.14, 2.04)	0.120
		Selection Up vs. Down	-0.009	(-0.25, 0.24)	0.958
		Selection Control vs. Down	0.017	(-0.25, 0.25)	0.864
		Selection Control vs. Up	0.027	(-0.26, 0.27)	0.804
	olfactory bulb	Brain weight	0.900	(-1.10, 2.46)	0.556
		Selection Up vs. Down	-0.008	(-0.14, 2.01)	0.108
		Selection Control vs. Down	0.009	(-0.22, 0.28)	0.944
		Selection Control vs. Up	0.018	(-0.22, 0.29)	0.864
	hypothalamus	Brain weight	0.793	(-0.24, 1.95)	0.160
		Selection Up vs. Down	-0.023	(-0.26, 0.22)	0.886
		Selection Control vs. Down	-0.013	(-0.23, 0.25)	0.948
		Selection Control vs. Up	0.010	(-0.23, 0.23)	0.902
Females	telencephalon	Brain weight	0.600	(-1.10, 2.40)	0.487
		Selection Up vs. Down	-0.011	(-0.28, 0.22)	0.894
		Selection Control vs. Down	-0.003	(-0.26, 0.28)	0.974
		Selection Control vs. Up	0.007	(-0.22, 0.26)	0.944
	optic tectum	Brain weight	0.565	(-0.99, 0.25)	0.486
		Selection Up vs. Down	0.008	(-0.24, 0.26)	0.912
		Selection Control vs. Down	-0.003	(-0.27, 0.22)	0.990
		Selection Control vs. Up	-0.011	(-0.28, 0.22)	0.930
	cerebellum	Brain weight	0.930	(-0.76, 0.28)	0.312
		Selection Up vs. Down	0.002	(-0.25, 0.29)	0.964
		Selection Control vs. Down	0.014	(-0.24, 0.26)	0.924

		Selection Control vs. Up	0.012	(-0.22, 0.27)	0.921
	dorsal medulla	Brain weight	0.558	(-0.18, 2.36)	0.530
		Selection Up vs. Down	-0.001	(-0.24, 0.27)	0.996
		Selection Control vs. Down	-0.007	(-0.27, 0.22)	0.983
		Selection Control vs. Up	-0.006	(-0.24, 0.24)	0.982
	olfactory bulb	Brain weight	0.643	(-1.00, 2.35)	0.502
		Selection Up vs. Down	-0.057	(-0.24, 0.20)	0.640
		Selection Control vs. Down	-0.033	(-0.27, 0.20)	0.774
		Selection Control vs. Up	0.023	(-0.22, 0.28)	0.854
	hypothalamus	Brain weight	0.603	(-1.34, 2.13)	0.520
		Selection Up vs. Down	-0.009	(-0.24, 0.24)	0.963
		Selection Control vs. Down	-0.001	(-0.30, 0.24)	0.992
		Selection Control vs. Up	0.008	(-0.27, 0.27)	0.942

Table S2: Average standard length of gonopodium selected ('down', control, 'up') male and female *G. holbrooki* across three replicates (in mm).

Treatment	Sex	Replicate	N	Mean \pm SE
down-selected	female	A	10	32.27 \pm 0.32
	female	B	10	31.00 \pm 0.22
	female	C	10	31.47 \pm 0.57
	male	A	10	23.89 \pm 0.72
	male	B	10	23.56 \pm 0.68
	male	C	9	24.77 \pm 0.59
control	female	A	10	32.20 \pm 0.39
	female	B	10	31.85 \pm 0.37
	female	C	10	32.98 \pm 0.35
	male	A	11	24.13 \pm 0.70
	male	B	10	24.11 \pm 0.48
	male	C	10	25.62 \pm 0.46
up-selected	female	A	10	32.22 \pm 0.34
	female	B	10	32.46 \pm 0.48
	female	C	10	32.33 \pm 0.24
	male	A	10	22.83 \pm 0.44
	male	B	10	23.15 \pm 1.00
	male	C	10	23.43 \pm 0.32

Table S3: Results from the best models (LMMs) on the full data set including one ‘down-selected’ female with a relatively large brain (see figure S1) are shown in grey-shaded cells (A). Results from the analysis excluding this data point are presented in white cells (B). All models used brain weight as the response variable, selection on gonopodium length as a fixed factor (the overall model also included sex as a fixed factor), body size as a co-variate and replicate nested within selection as a random effect. The response variable brain weight and the co-variate body size were log-transformed prior to analyses. Statistically significant results ($p < 0.05$) are highlighted in bold. Note that removing the outlier from our data set did not change our result qualitatively.

		(A) Analyses including one ‘outlier’			(B) Analyses excluding one ‘outlier’		
Model	Effects	<i>df (approx.)</i>	<i>F</i>	<i>p</i>	<i>df (approx.)</i>	<i>F</i>	<i>p</i>
Overall	Selection	2/9	1.482	0.276	2/9	1.450	0.283
	Sex	1/178	9.674	0.002	1/178	4.836	0.029
	Body size ¹	1/180	73.543	<0.001	1/177	66.083	<0.001
	Sex*Body size	1/178	9.832	0.002	1/178	4.969	0.027
Males	Selection	2/9	0.710	0.551	NA	NA	NA
	Body size ¹	1/173	896.0	<0.001	NA	NA	NA
Females	Selection	2/90	3.611	0.031	2/89	3.647	0.030
	Body size ¹	1/90	6.103	0.015	1/89	7.505	0.007

¹ log transformed Standard length

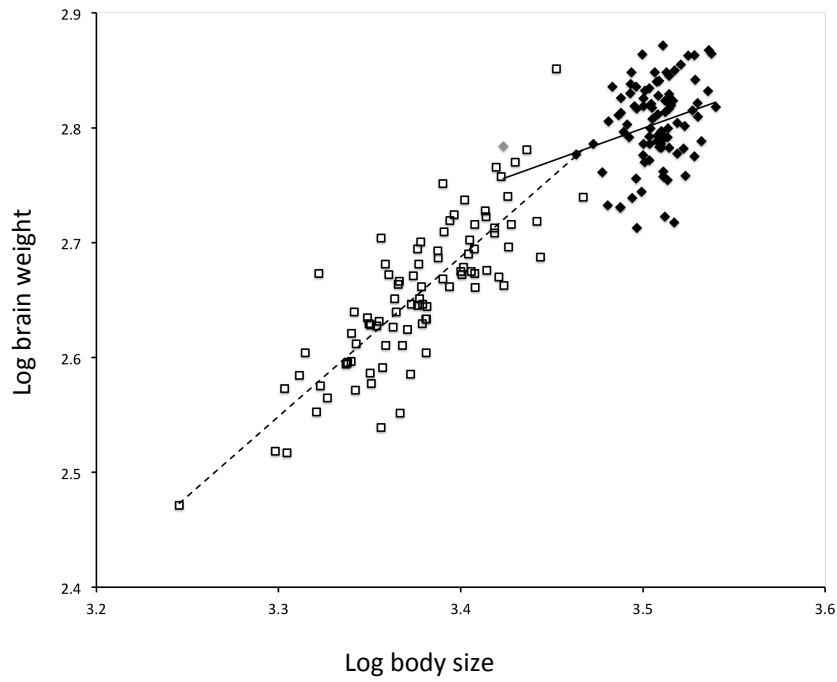


Figure S1: Relationship between body and brain size in male (open squares) and female (filled diamonds) mosquitofish (*G. holbrooki*). The grey diamond represents a down-selected female that is a potential outlier in this analysis. However, excluding it from our data does not change our results qualitatively (see table S3 for details on this).

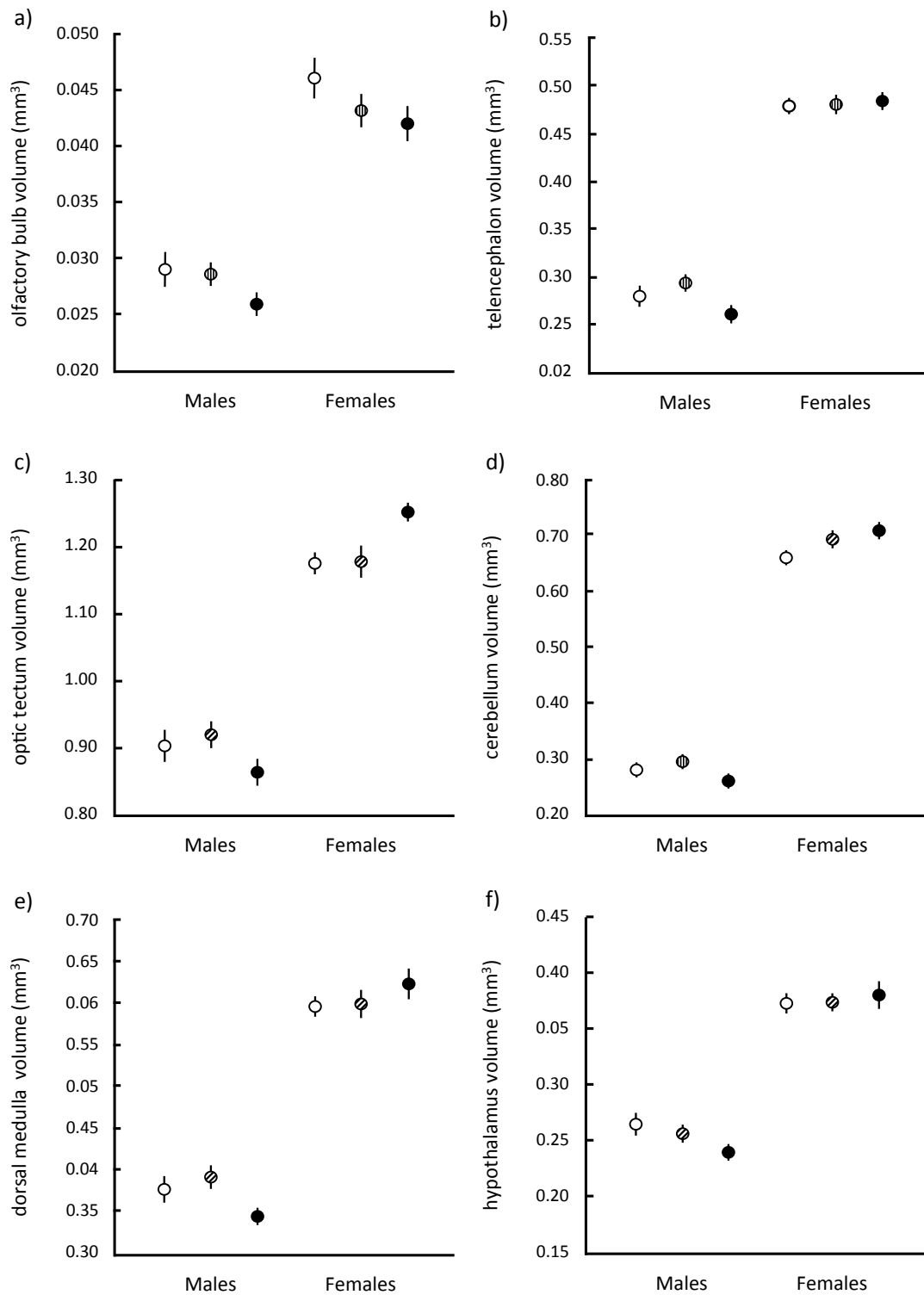


Figure S2 a-f: The volumes of the six brain subregions under study: (a) olfactory bulbs, (b) telencephalon, (c) optic tectum, (d) cerebellum, (e) dorsal medulla, and (f) hypothalamus for male and female mosquitofish ($n = 90$ for each sex) selected for relative gonopodium length (down, control, up) over eight generations. Open symbols show mean \pm SE for down-selected individuals, hatched symbols represent non-selected (control) individuals and filled symbols represent up-selected individuals ($n = 30$, for each experimental selection line and each sex). Note that that scaling for the Y-axis is different for each brain region.